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O-Oligosaccharidyl-1-amino-1-deoxyalditols as intermediates for fluorescent labelling of oligosaccharides

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Abstract—Reducing monosaccharides were efficiently converted to stable 1-amino-1-deoxyalditols (=glycamines; distinguished from glycosylamines by mass-spectrometry) during incubation at 20 °C in saturated aqueous NH_4HCO_3 containing $NaCNBH_3$. Potentially useful by-products included a novel, fully-reduced dimer (the corresponding secondary glycamine) and several relatively long-lived, unreduced products. With increasing incubation time, monomers exceeded dimers. Reducing disaccharides and oligosaccharides underwent similar reactions at their reducing termini; the yield of dimers decreased with increasing oligosaccharide M_r. The *O*-oligosaccharidyl-1-amino-1-deoxyalditols (OADs) obtained by reductive amination of oligosaccharides reacted readily with lissamine rhodamine sulfonyl chloride to yield OAD–sulforhodamine conjugates linked by a stable sulfonamide bond. Conditions for this reaction were optimised (borate buffer, pH 9.0–9.5). The highly fluorescent OAD–sulforhodamine products were purified on a C_{18} cartridge. They were electrophoretically immobile at pH 2.0 and 6.5, and migrated towards the anode in borate buffer, pH 9.4. The OAD–sulforhodamines were amenable to TLC and were excellent substrates for enzymic transglycosylation and for glycosylhydrolase action.

Keywords: 1-Amino-1-deoxyalditols; Dimeric glycamines; Fluorescent labelling; Glycamines; Reductive amination

1. Introduction

Oligosaccharides play many significant roles in biology. The availability of labelled oligosaccharides facilitates the study of their transport, binding, uptake, transglycosylation and degradation within living organisms as well as their modification by enzymes in vitro. Published oligosaccharide labelling strategies include

- incorporation of ^3H- or ^{14}C-labelled sugar residues into polysaccharides in vitro[1] or in vivo,[2] followed by enzymic fragmentation to yield radiolabelled reducing oligosaccharides;
- radiolabelling of the oligosaccharide's reducing terminus by reaction with NaB^3H_4, forming an [^3H]alditol group;[3,4]
- introduction of a UV-absorbing or fluorescent group by reductive amination of the reducing terminus with

Abbreviations: BAW, butan-1-ol/acetic acid/H_2O (12:3:5, unless otherwise stated); BPW, butan-1-ol/pyridine/H_2O (4:3:4); DMF, dimethylformamide; DNP-Lys, 2,4-dinitrophenyl-lysine; DP, degree of polymerisation; EAW, ethyl acetate/acetic acid/H_2O (10:5:6); LRSC, lissamine rhodamine sulfonyl chloride; m_{GlcN}, electrophoretic mobility relative to that of glucosamine (and corrected for electro-*endo*-osmosis); OAD, *O*-oligosaccharidyl-1-amino-1-deoxyalditol; PC, paper chromatography; PE, high-voltage paper electrophoresis; PyAW/CB, 1% (v/v) pyridine, 1% (v/v) acetic acid, 0.5% (w/v) chlorobutanol, pH 4.7; SR, sulforhodamine; XET, xyloglucan endotransglucosylase activity; XGO, xyloglucan-derived oligosaccharide (structure not specified); XLLG, XXLG, XXXG, XXFG, etc., specific xyloglucan-derived oligosaccharides (for nomenclature, see Ref. 22)

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$NaCNBH_3$ plus an aromatic amine such as 4-aminopyridine,[5] tyramine,[6] 8-aminonaphthalene-1,3,6-trisulfonic acid,[7] 2-aminoacridone,[8–11] or 2-amino-(6-amidobiotinyl)pyridine;[12] this approach can be used not only with aldoses but also with ketoses, which, however, yield an epimeric mixture;[13]

- radio-iodination of a previously introduced aromatic group.[14–16]

Although reductive amination with an aromatic amine is an efficient means of introducing specific groups, the choice of fluorescent labels is relatively restricted.

Less attention has been paid to the reductive amination of reducing oligosaccharides with inorganic ammonium salts to yield *O*-oligosaccharidyl-1-amino-1-deoxyalditols (OADs; *O*-oligosaccharidyl-glycamines; cf. Fig. 1). OADs are useful because they are readily amenable to further derivatisation at the stable primary amino group, for which purpose there are numerous labelling protocols. OADs are much more stable than *O*-oligosaccharidyl-glycosylamines, which are susceptible to hydrolysis, losing the amino group. Christiansen-Brams et al.[17] prepared OADs by reductive amination of cellobiose, lactose and maltose with benzylamine plus $NaCNBH_3$ followed by hydrogenolysis to remove the benzyl group. In a simpler, one-step method, an OAD was prepared by the reductive amination of lactose with $NaCNBH_3$ in aqueous ammonium ace-

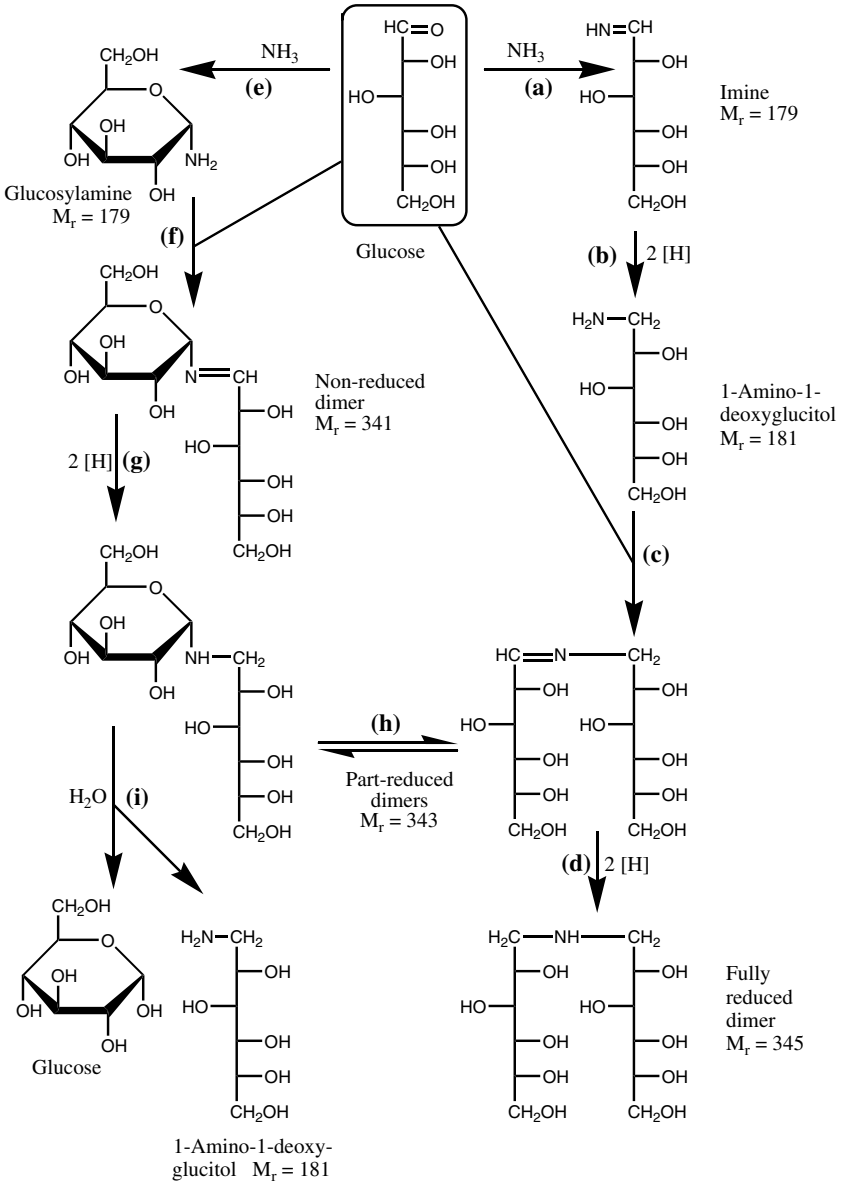

Figure 1. Proposed reaction of glucose with NH_4HCO_3 in the presence of reducing equivalents (2[H]) provided by $NaCNBH_3$. All the compounds shown, except glucose itself, are expected to be cationic at pH 2. The dimeric products are expected to predominate at relatively high glucose concentrations. The glucosylamine residues are arbitrarily shown in the α-pyranose ring form.

tate.[18] Another, high-yielding, method is to produce an oximine (by reaction of a reducing sugar with aqueous hydroxylammonium sulfate plus ammonium acetate) and then to convert this to the OAD by electro-reduction in a divided electrolysis cell with a working Hg cathode and a Pt-coated Ti anode separated by a cation-selective membrane.[19]

We have found that high yields of OADs are readily obtained with $NaCNBH_3$ in aqueous NH_4HCO_3, which is easier than ammonium acetate to remove by drying. We describe conditions for the reductive amination of sugars of various molecular weights by this method. The products include novel dimeric 'secondary glycamines' and probable higher oligomers. In addition, we report on a method for the subsequent labelling of OADs with the highly fluorescent sulforhodamine (SR) group and the use of the fluorescent conjugates as substrates for enzymes that act on saccharides.

2. Results and discussion

2.1. Reaction of monosaccharides and lactose with NH_4HCO_3 + $NaCNBH_3$

Incubation of non-radioactive monosaccharides (136 mM) with $NaCNBH_3$ plus NH_4HCO_3 at 20 °C resulted in the complete disappearance of the substrate and production of at least two lower-R_f products as resolved by paper chromatography (PC; data not shown). These products were ninhydrin-positive and stained weakly with $AgNO_3$, but failed to stain with aniline hydrogen–phthalate, indicating that they were aminated, non-reducing, sugar-derivatives. High-voltage paper electrophoresis (PE) confirmed that the non-reducing products were cationic at pH 2.0.

The use of radiolabelled sugars ([^{14}C]arabinose, [^{14}C]glucose and [^{14}C]lactose) allowed the detection of at least eight products (Fig. 2). In the cases of glucose and lactose, electrophoretically immobile ^{14}C-labelled material decreased with time, although some remained even after reaction times of 1 week, by which time no reducing sugar remained detectable by staining, possibly indicating the production of some ^{14}C-alditol. [Immobile ^{14}C-material diminished only slightly in the case of commercial '[^{14}C]arabinose' because much of the radioactivity was initially present in the form of an unreactive impurity, [^{14}C]arabinitol (see Section 3.2).]

With carrier-free ^{14}C-substrates (initial concentration ~0.1 mM), at least three strongly cationic products were rapidly formed (within the first 5 min); their yield remained almost unchanged during the first 24 h incubation. The major cationic products (which were also the fastest-migrating ones) formed within 24 h from arabinose, glucose and lactose had electrophoretic mobilities relative to that of glucosamine (m_{GlcN} values) of 1.10, 0.99 and 0.68, respectively. These values are thus inversely related to the M_r of the substrate, compatible with their being C_5, C_6 and C_{12} compounds, each with one fully charged $-NH_3^+$ group. Structures potentially accounting for these products include the imine, the 1-amino-1-deoxyalditol, and the anomeric glycosylamines, formed in reactions (a), (b) and (e), respectively (Fig. 1).

When non-radioactive carrier substrate was added, so that the initial substrate concentration was 9 or 90 mM instead of 0.1 mM, the yields of these products diminished; instead of them, slower-migrating products predominated, the major such products from arabinose, glucose and lactose having m_{GlcN} values of 0.73, 0.63 and 0.45, respectively (e.g., spot **E** in Fig. 2B). These mobilities suggest approximately half the charge:mass ratio of the corresponding products formed from carrier-free ^{14}C-substrates. This observation, and the fact that their formation was enhanced at high substrate concentrations, suggests that the lower-mobility products were formed from two sugar molecules [$(C_5)_2$, $(C_6)_2$ and $(C_{12})_2$] with one basic N atom. Proposed structures are shown in Figure 1.

These 'dimers' (or some of them) gradually reacted further. When the reaction was continued for 7 days, the lower-mobility products decreased in yield and were partially replaced by a single high-m_{GlcN} product in each case (e.g., compound **G** in Fig. 2B).

The preparation was repeated on a larger scale with 90 mM [^{14}C]glucose of low specific radioactivity, for incubation times of 6 h and 1 week. The products were subjected to preparative PE at pH 2.0, and the bands corresponding to the spots marked **A**–**H** (Fig. 2B) were eluted. The purity of each eluate was tested by re-electrophoresis of a small portion. Compounds **B**, **F** and **H** were thereby shown to be unstable, giving more than one peak of ^{14}C during the second PE run. The other compounds were stable (data not shown), and compounds **D**, **E** and **G** were recovered in sufficient amounts for MS.

Positive-mode MALDI–TOF mass-spectra of **D**, **E** and **G** each showed multiple m/z values, probably representing the compounds of interest plus non-radiolabelled impurities co-eluted from the paper. However, clear peaks were present that had m/z values expected for the predicted compounds of interest (cf. Fig. 1).

For example, compound **E** (the dimer present after 6 h incubation of glucose with NH_4HCO_3/$NaCNBH_3$) gave a peak at $m/z = 366$, corresponding to $M + Na^+$, where M is 343 ($C_{12}H_{25}NO_{10}$; required for the product that has been dimerised but not fully reduced). Compound **G** (the monomeric product present after 168 h incubation) gave a clear peak at $m/z = 204$, corresponding to $M + Na^+$, where M is 181 ($C_6H_{15}NO_5$; 1-amino-1-deoxyglucitol). It is possible that hydrolysis is responsible for conversion of one of the two isomeric, partially reduced dimers (**E**) to the fully reduced monomer (**G**)

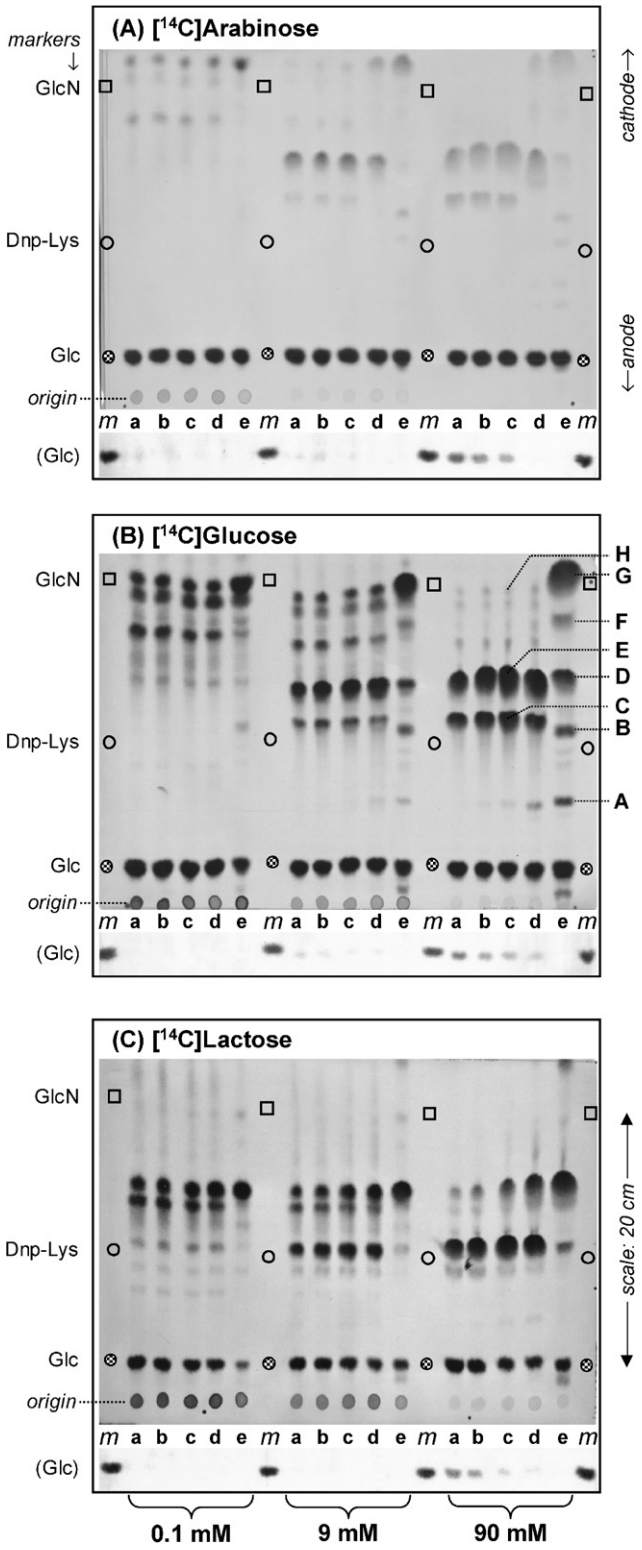

Figure 2. Electrophoresis of products formed by the action of $NaCNBH_3/NH_4HCO_3$ on reducing sugars. The substrate sugar was (A) [^{14}C]arabinose, (B) [^{14}C]glucose or (C) [^{14}C]lactose. The reaction was allowed to proceed for 0.1, 1, 6, 24 and 168 h (lanes a–e, respectively) with the substrate sugar initially present at 0.1, 9 and 90 mM (as indicated along the bottom). The products were resolved by PE at pH 2.0 (3 kV for 45 min) and detected by autoradiography (main images). The exact positions of non-radioactive markers (in '*m*' lanes), run on each electrophoretogram and stained after autoradiography, are plotted as follows: (□) glucosamine; (○) 2,4-dinitrophenyl-lysine; (▨) glucose. The narrow image below each autoradiogram shows the neutral compounds detectable after staining with aniline hydrogen–phthalate, which revealed the marker glucose (in '*m*' lanes) and the gradually disappearing substrate sugar (lanes a > b > c > d; visible in the 90 mM samples and faintly in the 9 mM samples). Note that neutral compounds migrate slightly from the origin owing to EEO. Spots **A**–**H** correspond to the compounds eluted from subsequent preparative electrophoretograms of the 6- and 168-h products obtained from 90 mM glucose.

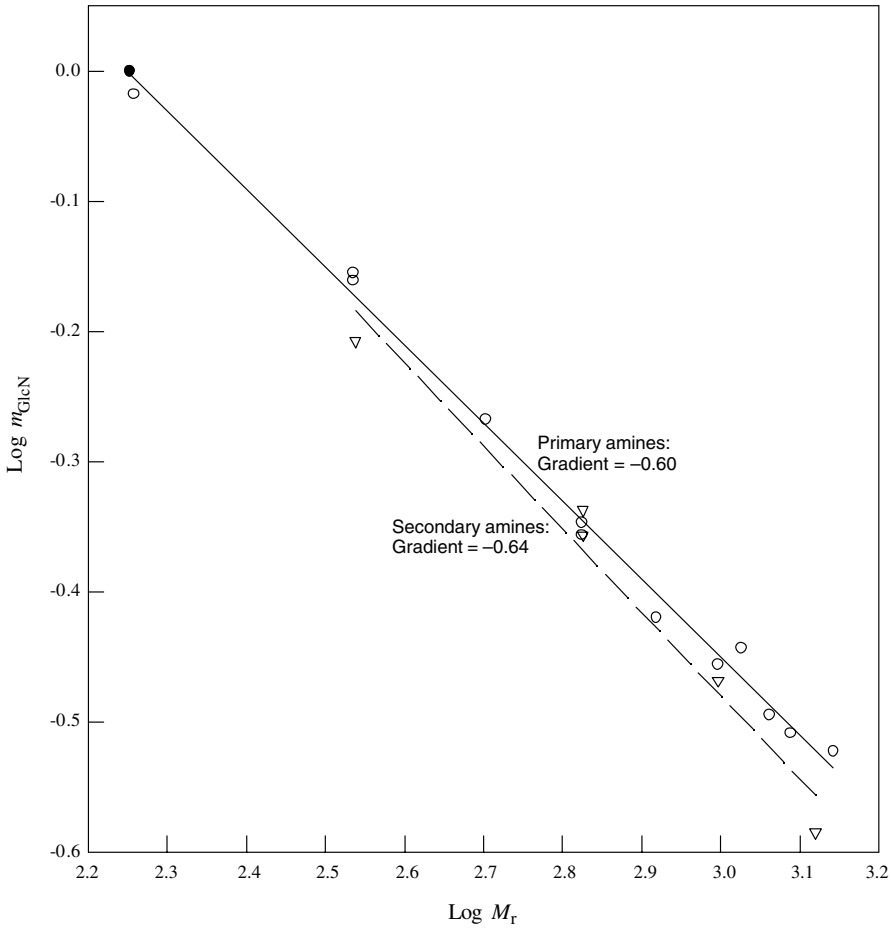

Figure 3. Electrophoretic mobilities (at pH 2.0) of the 1-amino-1-deoxyalditols obtained by reductive amination of glucose and several oligosaccharides with $NaCNBH_3/NH_4HCO_3$. Data-points for the monomeric aminodeoxyalditols (○) refer to those derived from (in order of decreasing m_{GlcN}) glucose, maltose, cellobiose, maltotriose, maltotetraose, isomaltotetraose, maltopentaose, maltohexaose, XXXG, maltoheptaose, XXLG and XLLG. Data for dimeric amines (▽) refer to those from (in order of decreasing m_{GlcN}) glucose, maltose, cellobiose, maltotriose and isomaltotetraose. The data-point for glucosamine itself is also plotted (●).

(Fig. 1; reaction (i)), which appears to occur between 6 and 168 h.

Compound **D** (a dimer still present after 168 h incubation) gave a peak at $m/z = 368.7$, corresponding approximately to $M + Na^+$, where M is 345 [$C_{12}H_{27}NO_{10}$; required for the fully reduced, stable dimer proposed to be formed in reaction (d) of Fig. 1].

Such dimers (**D** and **E**) do not appear to have been reported in previous studies of reductive amination. Potentially they represent valuable, novel, bifunctional carbohydrate derivatives.

2.2. Synthesis of OADs

When various oligosaccharides of DP > 4 [malto-oligosaccharides and xyloglucan-oligosaccharides (XGOs)] were incubated in $NaCNBH_3/NH_4HCO_3$, they gradually disappeared and were replaced by one major, cationic (as judged by PE), ninhydrin-positive, non-reducing sugar-derivative (OAD), which had a lower R_f on PC than the starting material. In experiments designed to optimise OAC production, the NH_4HCO_3 concentration was held constant (a saturated aqueous solution) and the concentration of $NaCNBH_3$ was varied: the rate of the reaction was maximal at the highest $NaCNBH_3$ concentration tested (640 mM; data not shown). High-M_r oligosaccharides (DP > 4) did not form ninhydrin-detectable dimers during reductive amination.

In the case of small oligosaccharides (DP 2–4; malto-oligosaccharides, cellobiose, isomaltotetraose), two products were formed. The lower-R_f product tended to be in the minority (unlike the situation with monosaccharides) and its yield decreased with increasing DP. On PE at pH 2.0, the lower-R_f product also had a lower m_{GlcN} than the higher-R_f product. These observations show that small oligosaccharides can form both monomeric and dimeric amines. This interpretation is supported by comparisons of electrophoretic mobility: a log–log plot of m_{GlcN} versus predicted M_r of the putative

monomeric amines was a straight line with a gradient of −0.60 (Fig. 3). This is a good approximation to the value ($-^2/_3$) predicted on the basis of Offord's model,[25] which states that the electrophoretic mobility of a molecule with a given charge is inversely proportional to the molecule's surface area, which can be approximated by $M_r^{2/3}$. Thus, these products were a series of differently sized oligosaccharide-derivatives, each with a single positive charge at pH 2.0, and an electrophoretic mobility approximated by $m_{GlcN} = k\varepsilon M_r^{2/3}$ Ref. 25, where k is a constant, ε is the net charge of the molecule (=+1), and M_r is the molecular weight. A log–log plot of the corresponding data for the putative dimeric amines (Fig. 3) gave a similar straight line, with a gradient of −0.64, in agreement with their proposed dimeric nature.

Quantitative studies of the reaction of low concentrations of a radiolabelled oligosaccharide with NH_4HCO_3 plus $NaCNBH_3$ showed a time-dependent production of the OAD (Fig. 4): after 6 days, the yield was 82% for the heptasaccharide [^{14}C]XXXG (Fig. 4a) and 83% for the nonasaccharide [^{3}H]XXFG (Fig. 4b). In the case of [^{14}C]XXXG, a minor, lower-m_{GlcN} product was also present at early sampling times (Fig. 4a), possibly representing a dimeric OAD; however, this product appeared to be converted into the major OAD during prolonged incubation, as was observed with monosaccharides and lactose (Fig. 2). The data show that the reductive amination of relatively large oligosaccharides (DP 7–9) under the conditions described was simple and efficient.

2.3. Sulforhodamine labelling of OADs

OADs should be ideal substrates for fluorescent labelling by condensation with any of the numerous amino-reactive probes such as LRSC. Condensation is expected to proceed best when the amino group of the OAD is un-ionised. This group is expected to have p$K_a \approx 9$–10. At pH > 10, the OAD should thus be most LRSC-reactive; however, such a high pH would also promote the unwanted hydrolysis of LRSC.

In a preliminary work, LRSC was found to condense with the OAD of the nonasaccharide, XLLG, much more completely in $NaHCO_3$–Na_2CO_3 buffer (pH 9) than in pure water. Alternative alkaline buffers were therefore also tested. The highest yields of XLLG–SR were obtained with borate or pyrophosphate buffer,

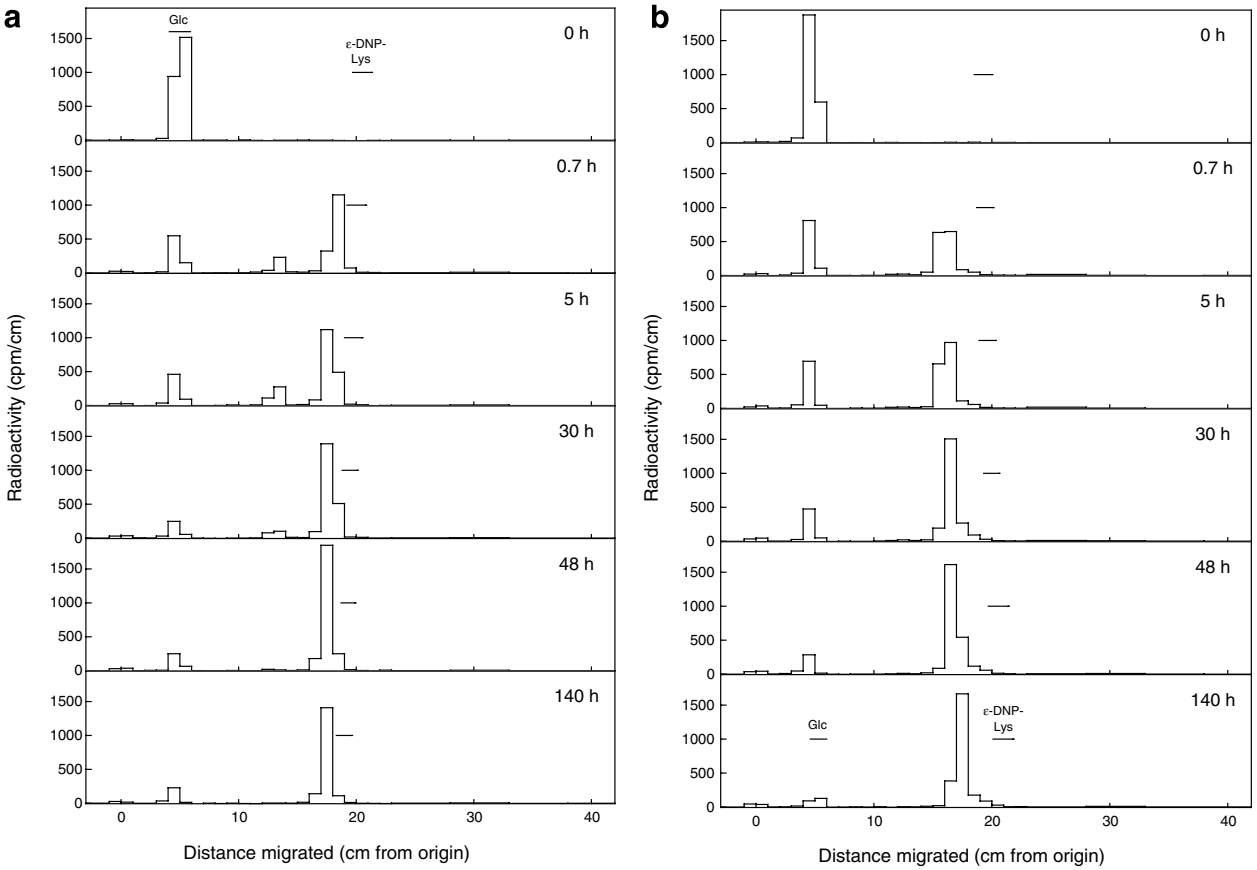

Figure 4. Reductive amination of XGOs with $NaCNBH_3/NH_4HCO_3$. Either 0.32 kBq of [^{14}C]XXXG (a) or 3.1 kBq of [^{3}H]XXFG (b) was incubated in 20 μL of 640 mM $NaCNBH_3$ in satd NH_4HCO_3 at 20 °C; 4-μL aliquots were sampled at intervals (0.7–140 h) and analysed by PE at pH 2.0. Glucose and N^{ε}-(2,4-dinitrophenyl)-lysine were used as markers (indicated by horizontal lines); the latter was an *internal* marker added to every radioactive sample.

pH 9.0–9.5; triethylamine buffer was not successful. Nevertheless, in each case, the majority of the OAD remained unreacted, as revealed by ninhydrin staining, and most of the LRSC was hydrolysed to free sulforhodamine. In a study of the optimum dose of LRSC, various volumes (20, 60, 180, 540 μL) of 1.3% LRSC in dimethylformamide (DMF) were added to 250-μL aliquots of 0.1% (w/v) OAD of maltoheptaose in 3% di-sodium tetraborate decahydrate (borax; pH 9.4). The optimum yield of heptasaccharide–SR conjugate (assessed by PC) was with 180 μL of the DMF solution. Excellent yields were, however, also obtained when LRSC was added as a dry powder rather than a DMF solution.

The same method of condensation (addition of LRSC in DMF to a solution of the OAD in 3% di-sodium tetraborate decahydrate, pH 9.4) was applied to a range of OADs. When the products were loaded on to a C_{18} BondElut column and rinsed with water (to remove DMF, borate and unreacted OAD), all the pink compounds remained adsorbed. Application of a methanol gradient eluted the latter, which were monitored by PC and TLC: 40–50% methanol eluted the free sulforhodamine plus a pink oligosaccharide derivative (**A**); 60% methanol eluted a second pink oligosaccharide derivative (**B**), uncontaminated by free sulforhodamine (Fig. 5). Essentially the same result was obtained for each OAD–sulforhodamine condensation attempted, except for those of acidic oligosaccharides, such as galacturonopentaose, whose condensation product was eluted from BondElut with 10% methanol.

For further purification, each '**B**' oligosaccharide–SR conjugate was dried on to filter paper and then rinsed in 96% ethanol. This eluted any traces of free sulforhodamine and other high-R_f contaminants without removing the oligosaccharide–SR conjugate; the latter was then eluted with water.

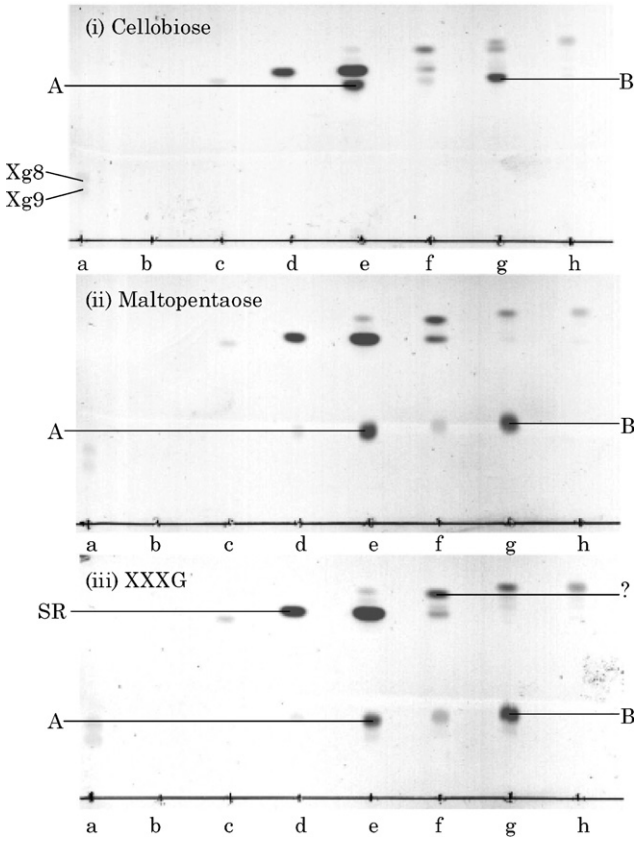

Figure 5. TLC of OAD–SR derivatives eluted from BondElut C_{18}.The OADs of three carbohydrates (i. cellobiose; ii. maltopentaose; iii. the heptasaccharide XXXG) were separately reacted with LRSC, then applied to 'BondElut' C_{18} columns. Material that remained bound to the columns in water was subsequently eluted with a step-gradient of methanol: b. 10%; c. 20%; d. 30%; e. 40%; f. 50%; g. 60%; h. 70%. From each eluate, 4 μL was subjected to TLC. Track (a) shows a marker mixture consisting of the OAD–sulforhodamine derivatives of XXLG (Xg8) and XLLG (Xg9). Each of the three OADs gave two major sulforhodamine derivatives, **A** and **B**. In addition, free sulforhodamine (SR; $R_F \approx 0.63$) and several minor products (e.g., marked '?') were eluted. The TLC was photographed under UV light; a negative of the image is shown so that fluorescent spots appear dark.

2.4. Chromatographic and electrophoretic properties of XGO–SRs

Conjugate **A** (see above) had a slightly lower R_f than conjugate **B** on both PC (Table 1) and TLC (Fig. 5). The difference in R_f was not great enough to suggest that **A** was a dimer—a difference that would have had a large effect on R_f (e.g., compare the R_f values of the SR derivatives of maltotriose and maltohexaose; Table 1 and Fig. 6). It is probable that **A** and **B** are isomers differing in the position of sulfation of the rhodamine moiety.

Both PC (Table 1) and TLC (Fig. 5) enabled a size-based separation of the SR-derivatives of OADs. Note, for example, the similarity of R_f of the derivatives of maltotetraose, isomaltotetraose and cellotetraose (Fig. 6). The SR-labelled products obtained from cellotetraose and cellopentaose had very low R_f values on PC owing to the affinity of the oligosaccharide moiety for cellulose by hydrogen-bonding; however, they migrated satisfactorily on silica gel TLC plates.

On PE (at pH 2.0 and 6.5), the SR conjugates of neutral oligosaccharides were immobile [relative to free glucose, the marker which indicates electro-*endo*-osmosis (EEO)]. However, as expected, the conjugate of $GalA_5$ had a net negative charge at pH 6.5. On PE in borate buffer (pH 9.4), all the conjugates moved towards the anode at rates governed by the affinity of the oligosaccharide moiety for borate (results not shown).

2.5. Use of sulforhodamine-labelled OADs as enzyme substrates

XLLG–SR is a useful acceptor substrate for xyloglucan endotransglucosylase (XET) activity.[26] Thus the modifi-

Table 1. Chromatographic and electrophoretic data for the sulforhodamine derivatives of various OADs

Sulforhodamine conjugate[a] of the OAD of	R_{SR} Value[b]					m_{G6P} Value[c]		m_{Man}[c]
	Product **A**, TLC	Product **B**, TLC	Product **A**, PC, BAW	Product **B**, PC, BAW	Products **A** + **B**, PC, BPW	Product **B**, PE, pH 2.0	Product **B**, PE, pH 6.5	Product **B**, PE, borate
Glucose	0.99	1.03	1.06	1.24	1.13	0.00	0.00	0.44
Maltose	0.92	0.93	0.74	1.00	0.95	0.00	0.00	0.39
Maltotriose	0.80	0.88	0.50	0.62	0.91	0.00	0.00	0.38
Maltotetraose	0.64	0.70	0.31	0.43	0.79	0.00	0.00	0.39
Maltopentaose	0.50	0.55	0.26	0.34	0.75	0.00	0.00	0.37
Maltohexaose	0.42	0.44	0.19	0.24	0.67	0.00	0.00	0.36
Maltoheptaose	0.34	0.38	0.06	0.09	0.61	0.00	0.00	n.d.
Isomaltotetraose	0.44	0.49	0.24	0.37	0.77	0.00	0.00	0.33
Cellobiose	0.91	0.96	0.75	0.91	0.99	0.00	0.00	0.40
Cellotetraose	0.52	0.63	0.07[str]	0.09[str]	0.29	—[pb]	—[pb]	0.27
Cellopentaose	0.33	0.38	0.00	0.00	0.03	—[pb]	—[pb]	—[pb]
XXXG	0.41	0.45	0.19	0.21	0.63	0.00	0.00	0.29
XXLG	0.31	0.34	0.14	0.16	0.53	0.00	0.00	0.36
XLLG	0.26	0.28	0.09	0.10	0.47	0.00	0.00	0.42
$GalA_5$	0.04	—	0.09	—	0.21	0.09	0.31	n.d.

[str]Streaked. [pb]Paper-binding: retained at or near the origin. n.d. = Not determined.

[a] The OAD of each oligosaccharide (or Glc) was reacted with LRSC in borate buffer and the products were fractionated on BondElut C_{18}. Each oligosaccharide (except $GalA_5$) yielded two sulforhodamine conjugates, **A** and **B** (see text).

[b] Chromatography systems were: TLC on silica gel in BAW (2:1:1); PC in BAW (12:3:5); and PC in BPW (4:3:4). R_{SR} = chromatographic mobility relative to that of free sulforhodamine; R_f values of sulforhodamine were 0.63 on TLC, 0.62 on PC in BAW, and 0.75 on PC in BPW. Products **A** and **B** were not well resolved by PC in BPW.

[c] Product **B** was subjected to PE at pH 2.0 [2 kV for 45 min, during which time glucose 6-phosphate (G6P) migrated 6.8 cm towards the anode with respect to the neutral marker, glucose], pH 6.5 (2 kV for 150 min; G-6-P migrated 20.7 cm towards the anode with respect to glucose), and in borate at pH 9.4 (2 kV for 150 min; mannose migrated 38 cm towards the anode with respect to 2,3,4,6-tetra-*O*-methylglucose). Abbreviations used: m_{G6P} = electrophoretic mobility relative to that of G6P, corrected for EEO by reference to glucose; m_{Man} = electrophoretic mobility relative to that of mannose, corrected for EEO by reference to 2,3,4,6-tetra-*O*-methylglucose.

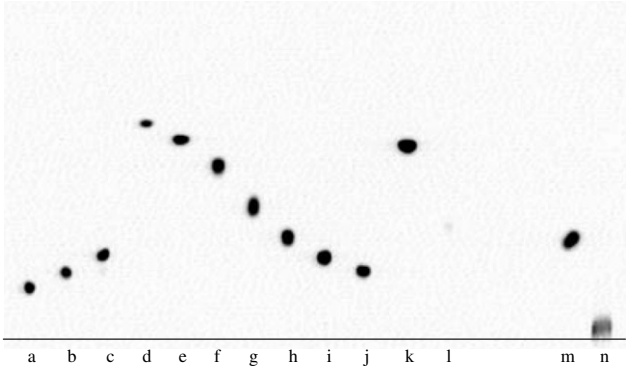

Figure 6. TLC of purified OAD–SR conjugates. The solvent was BAW (2:1:1). Parent compounds were (a) XLLG; (b) XXLG; (c) XXXG; (d) glucose; (e) maltose; (f) maltotriose; (g) maltotetraose; (h) maltopentaose; (i) maltohexaose; (j) maltoheptaose; (k) cellobiose; (l) cellotetraose; (m) isomaltotetraose; (n) galacturonopentaose.

cation of XLLG at its reducing terminus (involved in the conversion to XLLG–SR) does not prevent the non-reducing terminus acting as an acceptor substrate for enzymic transglycosylation. The fluorescence of the sulforhodamine group provided a very effective label by which XET activity can be detected and assayed.[27]

SR-labelled OADs are also promising substrates for the study of enzymes that hydrolyse oligosaccharides. For example, XLLG–SR is an effective substrate for the glycosidases (α-D-xylosidase, β-D-glucosidase and β-D-galactosidase) that degrade this XGO. TLC analyses show that XLLG–SR was gradually degraded by glycosidases present in extracts of many plant and fungal sources to yield products with higher R_f values (e.g., Fig. 7). For example, a crude extract from cauliflower rapidly removed one sugar residue from XLLG–SR, probably a β-Gal residue, and only much more slowly catalysed additional cleavage; in contrast, 'Driselase' eventually degraded XLLG–SR to the monomer, Glc–SR (Fig. 7). It is not possible to give a precise description of the course of oligosaccharide cleavage catalysed by these crude enzyme mixtures; however, the TLC illustrates the utility of this type of fluorescent conjugate as a glycosidase substrate. Enzyme-free controls showed no digestion (results not shown).

2.6. Conclusion

In this work, we have described the general ability of reducing monosaccharides and oligosaccharides to form cationic products during reductive amination with inorganic ammonium. After relatively long incubation times, the products appear to include 1-amino-1-deoxyalditols and OADs. After shorter incubation times, especially in the case of monosaccharides and low-DP oligosaccharides, dimeric products (secondary amines)

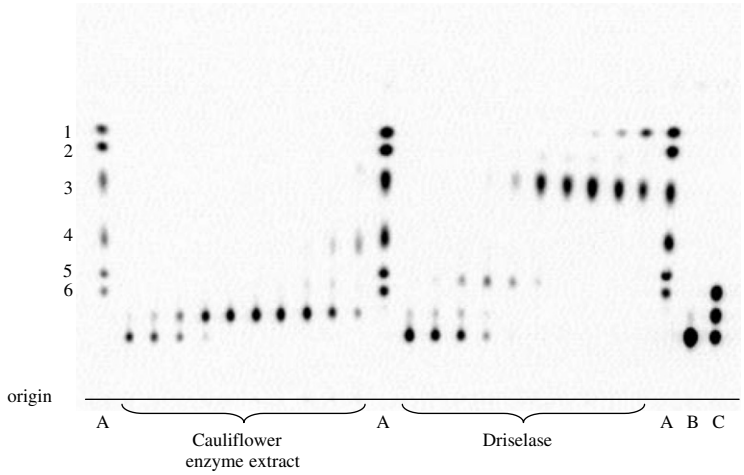

Figure 7. Use of OAD–SR conjugates as substrates for glycosylhydrolase activities. XLLG–SR (23 μM) was incubated at pH 4.7 with either a crude cauliflower extract or Driselase. After time intervals (4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 min; left to right), digestion was stopped with formic acid and 17 pmol (SR basis) of the substrate and/or products was subjected to TLC. The marker mixtures (A–C) contained the SR derivatives of (A) glucose (1) and maltose (2) to maltohexaose (6); (B) XLLG alone; (C) XLLG, XXLG and XXXG.

may predominate. The 1-amino-1-deoxyalditols and OADs appear to be stable derivatives, which, however, react with LRSC to form highly fluorescent oligosaccharide–SR conjugates. The conjugates thus formed are themselves stable and highly amenable to chromatographic and electrophoretic resolutions; they are valuable substrates for use in sensitive assays for transglycosylases and glycosidases.

3. Experimental

3.1. Electrophoresis and chromatography

High-voltage PE was conducted on Whatman 3MM paper. Unless otherwise stated, the buffer was pH 2.0 ($HCOOH/HOAc/H_2O$; 1:4:45, by vol) and runs were 3 kV for 45–65 min. Some PE was performed at pH 6.5 or in borate buffer (pH 9.4).[20] All PE mobilities are corrected for EEO by reference to a neutral marker. PC was performed by the descending method in solvents BAW ($BuOH/HOAc/H_2O$, 12:3:5, by vol), BPW ($BuOH/pyridine/H_2O$, 4:3:4, by vol) or EAW ($EtOAc/HOAc/H_2O$, 10:5:6, by vol). TLC was on silica gel in $BuOH/HOAc/H_2O$ (2:1:1, by vol, unless otherwise stated).

3.2. Source of XGOs and commercial ^{14}C-labelled sugars

The nonasaccharide XLLG ($Gal_2{\cdot}Xyl_3{\cdot}Glc_4$), octasaccharide XXLG ($Gal{\cdot}Xyl_3{\cdot}Glc_4$) and heptasaccharide XXXG ($Xyl_3{\cdot}Glc_4$) were prepared by enzymic digestion of *Tropaeolum* seed xyloglucan.[21] For abbreviated nomenclature of XGOs, see Ref. 22. The nonasaccharide XXFG ($Fuc{\cdot}Gal{\cdot}Xyl_3{\cdot}Glc_4$) was isolated by similar methods from cell walls of cultured rose cells, and catalytically tritiated as described[23,24] to yield [1-^{3}H]XXFG (~12 MBq/μmol) without loss of the reducing group. [^{14}C]XXXG was isolated by cellulase digestion of the cell walls of cultured sycamore (*Acer pseudoplatanus*) cells that had previously been grown for 12 days with D-[U-^{14}C]glucose (0.0067 MBq/μmol) as the sole carbon source: the [U-^{14}C]XXXG (~0.43 MBq/μmol) was purified by gel-permeation chromatography on Bio-Gel P-2 followed by two-dimensional preparative PC in EAW and BPW. The product was shown to be chemically pure by HPLC on Dionex CarboPac PA1,[21] and radiochemically pure by TLC.

D-[U-^{14}C]Glucose, D-[U-^{14}C]lactose were from Amersham, Bucks, UK, and were found to be of high radiochemical purity. The 'L-[1-^{14}C]arabinose' was from American Radiolabeled Chemicals Inc., St Louis; PC showed that this preparation was 38.2% radiochemically pure [^{14}C]arabinose, most of the ^{14}C being present as [^{14}C]arabinitol.

3.3. Reductive amination of reducing sugars

The reagent for reductive amination (640 mM $NaCNBH_3$ in satd aq NH_4HCO_3) was prepared immediately before use. For reductive amination of radiolabelled XGOs, 0.32 kBq [^{14}C]XXXG or 3.1 kBq [^{3}H]XXFG was dried, dissolved in 20 μL of reagent and incubated in a sealed vial in the dark at 20 °C; 4-μL aliquots were sampled at intervals (0.7–140 h). For radiolabelled mono- and disaccharides, 37 kBq of the sugar (D-[U-^{14}C]glucose, 'L-[1-^{14}C]arabinose' or D-[U-^{14}C]lactose) was dissolved in 120 μL of reagent either alone (giving a final sugar concentration of ~0.1 mM) or in the presence of enough of the corresponding non-radio-

active sugar to give a final concentration of 9 or 90 mM. The solution was incubated in a sealed vial in the dark at 20 °C; 10-μL aliquots were sampled at intervals. In each case, the sampled aliquots were applied to Whatman 3MM paper, air-dried for at least 24 h (without heating) to remove the majority of the NH_4HCO_3 and then subjected to PE at pH 2.0 and 3 kV (45 min for the ^{14}C-labelled monosaccharides and lactose; 65 min for the XGOs).

For the preparative scale, 70 μmol of a non-radioactive oligosaccharide (e.g., XLLG) was dissolved in 2.5 mL of reagent (containing ~1.7 mmol $NaCNBH_3$) and incubated in the dark at 20 °C for 7 days. The solution was then freeze-dried, re-dissolved in a minimal volume of H_2O and re-dried in a SpeedVac; the drying was repeated several times until there was no further loss of NH_4HCO_3.

3.4. Isolation of *O*-oligosaccharidyl-1-amino-1-deoxyalditols (OADs)

Two methods were then used for isolation of the OADs:

(a) The residue after removal of the majority of the NH_4HCO_3 was re-dissolved and passed through a 700-mL column of Bio-Gel P-2 in aq buffer [1% (v/v) pyridine, 1% (v/v) HOAc, 0.5% (w/v) chlorobutanol, pH 4.7 (PyAW/CB)]. Portions (5 μL) of each fraction were subjected to TLC on silica gel in BAW 3:1:1 and duplicate plates were stained with either ninhydrin or orcinol. In the case of XLLG, the major ninhydrin-positive compound eluted at $K_{av} = 0.26$, and had R_f 0.13 on TLC; unchanged XLLG eluted at K_{av} 0.34 and had R_f 0.17 on TLC. The ninhydrin-positive peak was dried, and 165-μg aliquots were subjected to PE at 3.5 kV for 75 min. The major spot had $m_{GlcN} = 0.34$ and stained with ninhydrin and $AgNO_3$ but not with aniline hydrogen–phthalate; a second, faint spot ($m_{GlcN} = 0.00$) stained with $AgNO_3$ but neither with ninhydrin nor aniline hydrogen–phthalate. The marker, N^{ε}-DNP-Lys, ran at $m_{GlcN} = 0.38$. PC of the OAD in EAW or BPW gave badly streaked spots. Final purification of the OAD was by preparative PE.

(b) As an alternative method for separation of OADs from unreacted oligosaccharides, the products from 10 mg oligosaccharide were re-dissolved in 1 mL H_2O, adjusted to pH ~ 4.8 with HOAc (indicator: bromocresol green), and passed through phosphocellulose (H^+ form; bed-volume 10 mL). Neutral sugars were eluted with 20 mL H_2O, then OADs with 10 mL of 150 mM TFA. The TFA was removed in vacuo. Aliquots of the residue were analysed by PE (pH 2.0, 3 kV for 45 min) and stained with $AgNO_3$.

3.5. Preparation of OAD–sulforhodamine conjugates (e.g., XLLG–SR)

A portion (50 mg) of the OAD of XLLG was dissolved in 3 mL of 3% (w/v) di-sodium tetraborate decahydrate (final pH = 9.1). A freshly prepared solution of 10 mg lissamine rhodamine sulfonyl chloride [LRSC; Molecular Probes Inc.] in 0.75 mL of dry DMF was added and incubated for 16 h in the dark. Another freshly prepared portion of 10 mg LRSC in 0.75 mL DMF was then added and incubated for a further 8 h. The reaction products were fractionated on Bio-Gel P-2 (bed-volume 80 mL; eluent PyAW/CB; 2.5-mL fractions were collected). A portion (5 μL) of each fraction was analysed by PC in BPW. Column fractions 8–13 contained XLLG–SR (giving an intense pink spot of $R_f \sim 0.42$ on PC). Fractions 12–13 yielded an additional pink spot (R_f 0.93). Fractions 7–13 contained the unreacted OAD (colourless; staining with ninhydrin). Fractions 13–50 (an intense peak, followed by a long tail) contained the hydrolysis product, lissamine rhodamine sulfonate (R_f 0.73). Fractions 8–12 were pooled and freeze-dried. The dark red solid was re-dissolved in 20 mL H_2O and passed through a 500-mg 'BondElut' C_{18} column (prewashed in 3 mL MeOH followed by 3 mL H_2O). All the pink material bound to top 1–2 mm of the column. A $H_2O \rightarrow$ MeOH gradient was applied, which eluted the XLLG–SR, monitored by PC in BPW and by TLC. The SR conjugates of other OADs were prepared similarly.

3.6. Sulforhodamine-labelled OADs as substrates for glycosylhydrolases

To 20 μL of 50 μM XLLG–SR was added 24 μL of PyAW/CB containing either 0.5% 'Driselase' (a cell wall-degrading preparation from the fungus *Irpex lacteus*, de-salted as described[20]) or a crude extract of cauliflower florets.[26] At intervals, 3-μL aliquots of the digests were transferred into 3 μL of 30% HCOOH, which stopped digestion, and 1.5 μL of the mixture was subjected to TLC.

3.7. Molecular mass determinations

M_r Determinations were carried out on a MALDI–TOF IV instrument (Shimadzu, Kratos Analytical, UK), which implemented a positive matrix-assisted laser desorption/ionisation coupled with in-source decay combined with a time-of-flight analyser. 2,5-Dihydroxybenzoic acid was used as the matrix. The sample was dissolved at 1 mg/mL in acetonitrile/H_2O (1:1). For the spot preparation, a mixture of 1 μL of the matrix with 10 pmol/μL analyte solution was used. The sample spots were air-dried at 20 °C. The ion acceleration voltage was set to 5 kV. Samples were irradiated by 337-nm photons

from a nitrogen laser. Typically, 100 shots were summed into a single mass-spectrum.

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